Angiostatic Steroids Potentiated by Sulfated Cyclodextrins Inhibit Corneal Neovascularization

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It is known that hydrocortisone can be converted to a potent angiogenesis inhibitor by coadministration with heparin or with a sulfated cyclodextrin. The activity of tetrahydrocortisol-S, a purely angiostatic corticosteroid, can be potentiated by beta-cyclodextrin tetradecasulfate as shown in this study. This drug "pair" and other pairs of corticosteroids and beta-cyclodextrin tetradecasulfate can be applied topically to inhibit corneal neovascularization. Endotoxin-induced corneal neovascularization in rabbits was treated with beta-cyclodextrin tetradecasulfate coadministered with either: hydrocortisone, tetrahydrocortisol-S, or 6-alpha-fluoro-17,21-dihydroxy-16-beta-methyl-pregna-4,9,(11)-diene-3,20-dione. When optimal ratios of steroid and cyclodextrin were used, neovascularization was reduced to 13%, 26%, and 28% of untreated controls for the three steroids, respectively. Hydrocortisone-cyclodextrin drug pairs suppressed virtually all inflammatory cell infiltration (induced by endotoxin), whereas tetrahydrocortisol-cyclodextrin pairs only partially reduced inflammation. These results demonstrate that corneal neovascularization and corneal inflammation are separable processes and that the neovascularization may be treated specifically using angiostatic steroids without antiinflammatory activity. Invest Ophthalmol Vis Sci 32:2898–2905, 1991

There are many conditions in which neovascularization of the cornea is undesirable. Uncontrolled corneal neovascularization is associated with scar formation, lipid deposition, immune rejection of corneal grafts, and subsequent blindness.1 Conventional therapy for corneal neovascularization relies mainly on antiinflammatory corticosteroids such as hydrocortisone, dexamethasone, and prednisolone. These steroids are thought to act by suppressing the recruitment of inflammatory cells (such as monocytes and macrophages) and inhibiting production or release of cytokines that recruit inflammatory cells.2,3

The neovascularization accompanying corneal inflammation can be induced by angiogenic factors such as tumor necrosis factor-alpha (TNF-α). Such factors are released by macrophages activated by endotoxin or other bacterial products.4-6 The use of antiinflammatory steroids for corneal neovascularization, however, may be complicated by cataract formation, microbial keratitis, and glaucoma. A more fundamental problem is that these steroids do not inhibit angiogenesis directly.7 Where inflammation is not the cause of angiogenesis, hydrocortisone per se has little or no effect on capillary growth. Examples of noninflammatory angiogenesis are tumor-induced neovascularization and developing vessels in the chick embryo chorioallantoic membrane.

We previously showed that hydrocortisone could be converted to a potent angiogenesis inhibitor by coadministration with heparin or fragments of heparin that lacked anticoagulant activity.7,8 We further found that certain corticosteroids, such as tetrahydrocortisol-S, could inhibit angiogenesis despite their lack of antiinflammatory activity.7 Tetrahydrocortisol-S is a naturally occurring relative of cortisone that has no glucocorticoid or mineralcorticoid activity.9 Its antiangiogenic activity also is potentiated by heparin.

However, the use of heparin as a potentiator of "angiostatic" steroids was problematic because its activity varied by batch and manufacturer.10 For the experimental treatment of corneal neovascularization, it was necessary to implant heparin into the corneal stroma because of the poor diffusibility of heparin through the cornea when applied topically as drops.

These problems were resolved by the discovery that certain low molecular-weight sulfated cyclodextrins...
can mimic heparin as a potentiator of angiostatic steroids and have little anticoagulant activity. Beta-cycloextrin tetradecasulfate is the most potent of these compounds tested so far and acts at concentrations 100 to 1000 times lower than heparin. It previously was shown that beta-cycloextrin molecules form an inclusion complex with steroids in which the steroid is carried in the hydrophobic cavity of beta-cycloextrin. The formation of this complex appears to be critical for the highly reproducible potentiation of angiostatic steroids by beta-cycloextrin. We now show that beta-cycloextrin tetradecasulfate can potentiate the antiangiogenic activity of natural and synthetic corticosteroids and that these angiostatic drug pairs are effective when applied topically to the cornea.

Materials and Methods

Animals

Male New Zealand albino rabbits (2–3 lb) were used in this study and handled in accordance with the ARVO Resolution on the Use of Animals in Research.

Endotoxin-Induced Corneal Neovascularization

Sustained-release polymers containing Escherichia coli endotoxin were implanted into rabbit corneas to induce corneal neovascularization. Weighed quantities of endotoxin (E. coli lipopolysaccharide; Sigma, St. Louis, MO) were mixed with ethylene vinyl acetate co-polymer (Elvax; DuPont) to create 1-mm³ pellets (2.0 ± 0.3 mg/pellet) containing 15% endotoxin (w/w). Endotoxin polymers were sterilized by ultraviolet irradiation before implantation.

We placed 155 rabbits under deep general anesthesia by intramuscular flank injection of 1:1 xylazine (Mobay, Shawnee, KS) and ketamine (Aveco, Fort Dodge, IA). The corneas were anesthetized topically with proparacaine (Alcaine; Allergan, Newport, CA). Dodge, IA). The corneas were anesthetized topically with a glass cannula and pushed to the distal end of the incision, and an intrastromal pocket was extended to 2 mm from the limbus. Polymer pellets of uniform weight and containing endotoxin were introduced with a glass cannula and pushed to the distal end of the pocket with a Teflon (Wilbur Scientific, Boston, MA) trochar. The iris spatula then was applied to the corneal surface to appose the two layers of split stroma. The eyes were treated with a single dose of topical erythromycin and allowed to heal for 48 hr before examination.

Topical Angiostatic Preparations

Hydrocortisone: Hydrocortisone-21-phosphate (0.5 mg/ml; Sigma) was dissolved in 1.5% methylcellulose. To this concentration of hydrocortisone solutions, beta-cycloextrin tetradecasulfate (Takeda, Osaka, Japan) was added so that its final concentration was 0.25 mg/ml, 0.5 mg/ml, 1.0 mg/ml, or 2.0 mg/ml. All solutions were vortexed vigorously with sterile glass beads at the time of initial mixing and just before application. The solutions were stored at 4°C for at least 48 hr before use.

Tetrahydrocortisol-S: Tetrahydrocortisol-S (1 mg/ml; Sigma) was suspended in the 1.5% methylcellulose vehicle. Beta-cycloextrin (0.5 mg/ml, 1.0 mg/ml, 2.0 mg/ml, or 4.0 mg/ml) was added to tetrahydrocortisol-S solutions, and these suspensions were vortexed with sterile glass beads and stored as described.

U-72, 745G: The synthetic steroid U-72, 745G (6-alpha-fluoro-17,21-dihydroxy-16-beta-methyl-pregn-4,9,(11)-diene-3,20-dione 1 mg/ml; Upjohn, Kalamazoo, MI) was dissolved in the 1.5% methylcellulose vehicle. Beta-cycloextrin tetradecasulfate (0.5 mg/ml, 1.0 mg/ml, 2.0 mg/ml, or 4.0 mg/ml) was added to these solutions and stored as described.

Treatment Protocol

Droppers were made by attaching a 20-gauge intravenous catheter (Cathlon IV; Crimkon, Tampa, FL) to a 3-ml syringe (Becton Dickinson, Rutherford, NJ). Angiostatic drug solutions (0.5 ml) were applied to each eye three times daily for 13 days, beginning on day 3 after implantation of the endotoxin pellet. Control eyes received vehicle only or no treatment.

Slit-Lamp Biomicroscopy

Corneas were examined every other day, using a Zeiss slit-lamp stereoscope at 10X, for 16 days after surgery. A calibrated micrometer was used to measure the length of corneal vessels growing toward the implant.

India-Ink Injection of Corneal Vessels

Sixteen days after implantation of the endotoxin pellets, the rabbits were placed under deep general anesthesia with intramuscular ketamine (43.5 mg/kg) and xylazine (8.7 mg/kg). A cannula was inserted into the carotid artery ipsilateral to the cornea to be injected. The cannula was infused with heparin (1000 U), and then the animals were killed with an overdose of intravenous phenobarbital. The cannula was flushed with 360 ml of lactated Ringer’s solution until the corneal vessels were clear of visible blood. Then
Fig. 1. Topical beta-cyclodextrin tetradecasulfate alone (1 mg/ml) does not inhibit corneal neovascularization in rabbit eyes (n = 15), when vessel length is measured by slit-lamp biomicroscopy. Increased vessel density was observed in all treated eyes.

240 ml of 10% India ink in 11% gelatin solution, warmed to 37°C, was injected into the catheter, until corneal vessels were filled completely with ink.16

The corneas were frozen immediately in situ with pressurized Freon (Dust-Away; Satter, Denver, CO). The eyes were enucleated and fixed in chilled 10% phosphate-buffered formalin. The corneas were dissected away from the globe under cold conditions and photographed.

Histologic Examination

The corneas with India ink-injected vessels were fixed in 10% phosphate-buffered formalin. The specimens were embedded in paraffin, sectioned, and stained with hematoxylin and eosin, Masson trichrome, and periodic acid-Schiff.

Results

Implantation of sustained-release endotoxin polymers in rabbit corneas stimulated new vessel growth into the cornea at a mean rate of 0.21 ± 0.12 mm/day. Maximal inhibition of corneal vessels by the angiostatic drug pairs occurred after approximately 9 days of treatment, although the earliest response was evident within 2 days of therapy. The vehicle alone did not inhibit angiogenesis. Topical hydrocortisone alone (0.5 mg/ml) reduced the rate of vessel growth to 76% of untreated control corneas. Topical tetrahydrocortisol-S alone and steroid U-72, 745G (each at 1.0 mg/ml) inhibited vessel growth to 47%. Topical beta-cyclodextrin tetradecasulfate alone (1.0 mg/ml) did not inhibit angiogenesis (Fig. 1) and, in all cases, increased the density of neovascularization as observed by slit-lamp biomicroscopy. However, beta-cyclodextrin tetradecasulfate potentiated the effect of angiostatic steroids (Fig. 2). The hydro-

Fig. 2. Beta-cyclodextrin potentiates the angiostatic activity of three steroids (A = hydrocortisone; B = tetrahydrocortisol-S; C = U-72, 745G) when topically administered in its optimal steroid: cyclodextrin ratio to rabbit corneas containing sustained-release endotoxin pellets. Topical treatment was not initiated until 3 days after implantation of the endotoxin pellet to allow neovascularization to occur.
cortisone–beta-cyclodextrin drug pair reduced vessels to 13% of untreated control eyes. The tetrahydrocortisol–beta-cyclodextrin drug pair inhibited vessels to 26% of untreated controls. The U-72, 745G–beta-cyclodextrin drug pair reduced angiogenesis to 28% of untreated controls. The drug pairs profoundly decreased both vessel density and caliber, with the greatest effect seen at the growing tips of vessels. In these experiments, we quantified only the vessel length, recognizing that measurement of vessel length alone underestimated the full extent of angiogenesis inhibition.

Increasing ratios of steroid–beta-cyclodextrin (w/w) drug combinations were tested initially, e.g., 1:0.5, 1:1, 1:2, and 1:4 (Fig. 3). For each steroid, an optimal ratio showed maximal inhibition of angiogenesis. For hydrocortisone, the optimal steroid–cyclodextrin ratio was 1:2. For tetrahydrocortisol, the optimal ratio was 1:0.5. For U-72, 745G, ratios of either 1:2 or 1:4 were optimal. Regression of neovascularization was observed only in eyes treated with an optimum steroid–cyclodextrin drug pair. Our criteria for vessel regression consisted of observing the following: (1) significantly decreased density of neovascularization; (2) diminishing measurements of vessel length; and (3) ghost tracks of no-perfused vessels (Fig. 4, Panel D). Drug pairs that varied from the optimal ratio showed diminished or no antiangiogenic activity.

Histologic sections of untreated corneas revealed capillaries and larger vessels in the stroma extending from the limbus toward the endotoxin–polymer implant. A dense inflammatory cell infiltrate invaded the edge of the corneal pocket adjacent to the polymer. Leukocytes invaded the cornea and migrated from the limbus to the endotoxin pellet. The corneal epithelium and endothelium appeared normal (Fig. 5).

Corneas treated with optimal doses of hydrocortisone and cyclodextrin in the optimal ratio had no vessels in the stroma and virtually absent inflammatory cells. Those treated with optimal tetrahydrocortisol and cyclodextrin also had no vessels in the stroma. However, an inflammatory cell infiltrate persisted at the edge of the pocket, although it was decreased compared with untreated controls (Fig. 5).

**Discussion**

These studies show that topical coadministration of an angiostatic steroid with beta-cyclodextrin tetradecasulfate effectively inhibits corneal neovascularization and causes regression of new vessels. Corneal neovascularization was induced by the sustained-release of *E. coli* endotoxin. Endotoxin stimulates an inflammatory infiltrate rich in macrophages activated to release various cytokines including TNF-α, a potent angiogenic factor. Hydrocortisone alone reduced neovascularization to 76% of untreated controls. The virtual absence of inflammatory cells in histologic sections of these corneas supports the view that partial inhibition of neovascularization by hydrocortisone alone may be a result of its antiinflammatory effect. The inhibition was potentiated 3.6-fold by coadministration with beta-cyclodextrin tetradecasulfate, reducing the neovascularization to 13%.
Fig. 4. India ink injections of rabbit corneas 16 days after implantation of a sustained-release endotoxin pellet 2.0 mm from the limbus. Treatment was initiated when the vessels had reached a mean length of 0.3 mm from the limbus, and at this time, more than ten vessels could be counted. (a) Untreated. Vessels surround the pellet and there are focal hemorrhages. (b) Hydrocortisone alone, 13 days of therapy. Vessels reach but do not completely encircle the endotoxin pellet. (c) Beta-cyclodextrin tetradecasulfate alone. Vessels have encircled the endotoxin pellet and the advancing front is wider and more dense than the untreated cornea. (d) Hydrocortisone and beta-cyclodextrin tetradecasulfate (1:2 ratio). Corneal vessels were inhibited and never reached the endotoxin pellet. Furthermore, many vessels underwent regression (see arrows). This was seen as a significant decrease in the number of vessels, and by decreased vessel length when compared to the first day of treatment.
Fig. 5. Histologic sections of rabbit corneas 16 days after implantation of endotoxin pellet (Masson trichrome stain) (a) Untreated. New vessels filled with India ink (arrows) and a florid inflammatory infiltrate have reached the corneal pocket (P) which contains the endotoxin pellet (×80). (b) Hydrocortisone and beta-cyclodextrin tetradecasulfate (1:2 ratio). No vessels have reached the corneal pocket and there are virtually no inflammatory cells (×80). (c) Tetrahydrocortisol and betacyclodextrin tetradecasulfate (1:0.5 ratio). No vessels have reached the corneal pocket. There is a mild inflammatory infiltrate contiguous to the corneal pocket (×80).
contrast, tetrahydrocortisol-S alone inhibited corneal neovascularization to 47% of untreated controls, despite the presence of an inflammatory infiltrate. This result was consistent with our previous finding that tetrahydrocortisol-S is an angiostatic steroid with no glucocorticoid or mineralocorticoid activity. Co-administration with beta-cyclodextrin tetradeacasulfate increased the antiangiogenic activity of tetrahydrocortisol-S by 1.4-fold, reducing corneal neovascularization to 26% of untreated controls. A mild inflammatory cell infiltrate was present. The angiostatic steroid U-72, 745G is a synthetic analogue of dexamethasone with decreased antiinflammatory activity. Its angiostatic effect similarly is potentiated by beta-cyclodextrin tetradeacasulfate. These results show that angiogenesis can be inhibited independently of inflammation. Angiogenesis and inflammation thus appear to be separable processes.

We would like to emphasize that the discovery of angiostatic steroids without glucocorticoid action raises the possibility that corneal neovascularization and corneal inflammation may be treated separately and specifically. Furthermore, the angiostatic activity of these steroids can be potentiated by coadministration of beta-cyclodextrin tetradeacasulfate. We propose that in treating some forms of corneal neovascularization, it may be imprudent to use an antiinflammatory steroid at its toxic limit to attain at best, minimal inhibition of angiogenesis. A more effective therapy would be to use a purely angiostatic steroid. An antiinflammatory steroid then could be added, according to the extent of inflammation present.

It is not known whether the complications of cataract formation, microbial keratitis, and glaucoma associated with hydrocortisone, prednisolone, and other antiinflammatory corticosteroids will be obviated by either tetrahydrocortisol-S (or its analogues) or by the reduced concentrations of hydrocortisone required (0.5 mg/ml steroid used in our studies compared with 10 mg/ml steroid in conventional clinical preparations) when complexed with beta-cyclodextrin tetradeacasulfate. It is known that steroids enter the hydrophobic cavity of cyclodextrin and form inclusion complexes.\(^{11,19}\) The optimum weight–weight ratios we used provided steroid in excess of cyclodextrin when complexed with beta-cyclodextrin tetradeacasulfate. It is known that steroids enter the hydrophobic cavity of cyclodextrin and form inclusion complexes.\(^{11,19}\) The optimum weight–weight ratios we used provided steroid in excess of cyclodextrin in a 3:1 (hydrocortisone) or 6:1 (tetrahydrocortisol-S and U-72, 745G) molar ratio of steroid to cyclodextrin. A similar ratio dependency was observed for steroid–cyclodextrin pairs that inhibited migration of capillary endothelial cells in vitro.\(^{12}\) Inhibition of endothelial cell migration increased in proportion to the time of mixing of the steroid and cyclodextrin before treatment of endothelial cells. Biologic activity of the drug pair was maximal after 48 hr of mixing at 4°C. In our studies, steroids and beta-cyclodextrin tetradeacasia sulfate were mixed at least 48 hr at 4°C before application to rabbit corneas, and such a preparation time for the purpose of complex formation may be important if these drug pairs are to be used clinically. Furthermore, when the optimum ratio of beta-cyclodextrin tetradeacasulfate to steroid was exceeded by excess cyclodextrin, the antiangiogenic effect was reduced. For example, when combined with hydrocortisone (0.5 mg/ml), beta-cyclodextrin concentrations greater than 2.5 mg/ml and up to 20 mg/ml were no longer inhibitory.

The optimum concentrations of angiostatic steroids and beta-cyclodextrin tetradeacasulfate caused regression of proliferating blood vessels. For example, all eyes receiving an optimum steroid–cyclodextrin drug pair showed marked regression in vessel density 2 days after treatment initiation. Vessel length also regressed in many of these eyes, and this was greatest for the hydrocortisone–cyclodextrin pair. In this case, by the 9th treatment day (day 12 postsurgery), the vessels had regressed by 50% of their peak length (as measured on day 6 postsurgery; Fig. 2, Panel A). Therefore, vessel regression may be an additional advantage of this form of angiostatic therapy that is not observed usually with conventional steroid therapy. It is not clear whether the regression of these corneal vessels is by the same mechanism we previously reported for a different model.\(^{20}\)

Our recent studies show that topically administered complexes of steroid and beta-cyclodextrin tetradeacasulfate (radiolabeled) accumulate in the cornea and are released in a sustained fashion into the aqueous humor.\(^{21}\) Thus, it is conceivable that topical angiostatic therapy may be useful in treating neovascular glaucoma.

**Key words:** hydrocortisone, beta-cyclodextrin tetradeacasulfate, angiostatic steroids, corneal neovascularization, angiogenesis inhibition

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